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Cytoplasmic Relocalization of Heterogeneous Nuclear Ribonucleoprotein A1 Controls Translation Initiation of Specific mRNAs[D]

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Heterogeneous nuclear ribonucleoprotein (hnRNP) A1 is a nucleocytoplasmic shuttling protein that regulates gene expression through its action on mRNA metabolism and translation. The cytoplasmic redistribution of hnRNP A1 is a regulated process during viral infection and cellular stress. Here, we show that hnRNP A1 is an internal ribosome entry site (IRES) *trans*-acting factor that binds specifically to the 5′ untranslated region of both the human rhinovirus-2 and the human apoptotic peptidase activating factor 1 (apaf-1) mRNAs, thereby regulating their translation. Furthermore, the cytoplasmic redistribution of hnRNP A1 after rhinovirus infection leads to enhanced rhinovirus IRES-mediated translation, whereas the cytoplasmic relocalization of hnRNP A1 after UVC irradiation limits the UVC-triggered translational activation of the apaf-1 IRES. Therefore, this study provides a direct demonstration that IRESs behave as translational enhancer elements regulated by specific *trans*-acting mRNA binding proteins in given physiological conditions. Our data highlight a new way to regulate protein synthesis in eukaryotes through the subcellular relocalization of a nuclear mRNA-binding protein.

INTRODUCTION

Although some evidence exists that translation may take place within the nucleus (Iborra *et al*., 2001, 2004; Brogna *et al*., 2002; Dahlberg and Lund, 2004), it is generally accepted that translation occurs in the cytoplasm (Gebauer and Hentze, 2004). Strikingly, however, a number of predominantly nuclear mRNA binding proteins, known for their functions in nuclear pre-mRNA splicing, were recently identified as *trans*-acting factors controlling translation initiation of specific mRNAs. These proteins, which constitute the most abundant cellular proteins contributing to messenger ribonucleoprotein particle formation, are members of the splicing regulator (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) classes. Recent work has revealed an unexpected role of several members of the SR protein family, including ASF/SF2 and SRp20, in translational control (Sanford *et al*., 2004; Bedard *et al*., 2007). Among the hnRNP family, hnRNP K and hnRNP E1 silence translation of the 15-lipoxygenase (LOX) mRNA in immature erythroid precursor cells (Ostareck *et al*., 1997). Several hnRNPs, including A1, C1/C2, E1/E2, I (PTB), and L, are independently involved in the translational control of specific mRNAs containing internal ribosome entry sites (IRESs) through a cap-independent mechanism (for review, see Vagner *et al*., 2001; Bonnal *et al*., 2003; Komar and Hatzoglou, 2005). These hnRNP proteins constitute IRES *trans*-acting factors (ITAFs) that modulate the activity of IRES sequences generally present in the 5′ untranslated region (UTR) of several viral and cellular mRNAs (Spriggs *et al*., 2005).

Most of these predominantly nuclear regulators are able to shuttle between the nucleus and the cytoplasm associated with their target mRNAs (Piñol-Roma and Dreyfuss, 1992; Caceres *et al*., 1998; Mili *et al*., 2001). This has led to the assumption that these mRNA binding proteins might translocate to the cytoplasm as preformed complexes with their target mRNAs to regulate translation. This hypothesis has been supported by several reports proposing that the lack of IRES activity after RNA transfection might indicate that an IRES-containing mRNA requires a "nuclear experience" to become active (i.e., Stoneley *et al*., 2000). In these cases, the transfected RNAs remain in the cytoplasm; therefore, they would be unable to meet their cognate nuclear *trans*-acting

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factors. However, a definitive proof for this hypothesis is missing because of the lack of proper experimental design to force the RNA to enter the nucleus upon transfection.

Another intriguing possibility is that these translational *trans*-acting factors relocalize to the cytoplasm before they are able to bind to their cognate mRNAs. One advantage of this model is that it provides another layer of translational regulation through subcellular relocalization of *trans*-acting factors. Such regulation has been demonstrated for splicing events, because stress-induced relocalization of hnRNP A1 to the cytoplasm changes alternative splicing patterns of splicing reporters in cell culture (van der Houven van Oordt *et al*., 2000).

Here, we have studied the link between the subcellular localization of an mRNA binding protein and its ability to control translation initiation. We have focused our study on hnRNP A1, because hnRNP A1 is a shuttling protein that was shown to be associated with poly(A)+ mRNA in the cytoplasm (Piñol-Roma and Dreyfuss, 1992; Mili *et al*., 2001). hnRNP A1 can act as a general RNA binding protein in mediating cap-dependent translation inhibition (Svitkin *et al*., 1996). However, because hnRNP A1 can also function as a sequence-specific RNA binding protein (Burd and Dreyfuss, 1994), it has been a long standing hypothesis that hnRNP A1 could be a translational regulator of specific mRNAs (Piñol-Roma and Dreyfuss, 1992; Svitkin *et al*., 1996). Consistent with this hypothesis, we have recently shown that hnRNP A1 promotes IRES-mediated translation of the human fibroblast growth factor (FGF)-2 mRNA and inhibits the IRES-mediated translation of the X-linked inhibitor of apoptosis (XIAP) mRNA (Bonnal *et al*., 2005; Lewis *et al*., 2007). Importantly, several findings demonstrate that hnRNP A1 can be relocalized to the cytoplasm in a regulated manner. For example, infection of cells with picornaviruses, including the human rhinovirus (HRV), leads to proteolysis of specific components of the nuclear pore complex, which in turn results in the cytoplasmic relocalization of several proteins, including hnRNP A1 (Gustin and Sarnow, 2001). Because the replicative cycle of picornaviruses is entirely carried out in the cytoplasm of the infected host cell, it is possible that the cytoplasmic redistribution of hnRNP A1 modulates the activity of the rhinovirus IRES. Furthermore, activation of the mitogen-activated protein kinase(3/6)-p38 signaling pathway in mammalian cells stressed by osmotic shock or UVC irradiation results in phosphorylation-dependent cytoplasmic accumulation of hnRNP A1 (van der Houven van Oordt *et al*., 2000; Allemand *et al*., 2005). Interestingly, UVC-dependent IRES-mediated translational control has recently been demonstrated for the apoptotic peptidase activating factor 1 (apaf-1) mRNA (Ungureanu *et al*., 2006), which encodes a proapoptotic protein involved in the formation of the apoptosome (Schafer and Kornbluth, 2006).

The above-mentioned observations prompted us to examine the involvement of hnRNP A1 in HRV and apaf-1 IRES-mediated translation and to investigate the influence of the cytoplasmic relocalization of hnRNP A1 on HRV and apaf-1 IRES activities. We report that both the HRV IRES and the apaf-1 IRES bind hnRNP A1. We show that hnRNP A1 has an opposite effect on these IRESs, because it activates HRV IRES activity but inhibits apaf-1 IRES-mediated translation. The cytoplasmic redistribution of hnRNP A1 after rhinovirus infection leads to enhanced HRV IRES activity in the cytoplasm, whereas the cytoplasmic relocalization of hnRNP A1 after UVC irradiation limits the UVC-triggered translational activation of the apaf-1 IRES in the cytoplasm. Together, our data demonstrate that the cytoplasmic relocalization of hnRNP A1 is pivotal in regulating IRES-mediated translation after rhinovirus infection and UVC-triggered apoptosis.

MATERIALS AND METHODS

Plasmid Constructs and Primers

See Supplemental Figure S4 and Supplemental Table S4 for primer sequences used for cloning. Sequences of primers used to generate polymerase chain reaction (PCR) DNA templates for in vitro transcription are found in Supplemental Table S5.

Cell Culture, Rhinovirus Infection, and UVC Irradiation

The human rhinovirus was incubated 1 h with HeLa cells plated on 24-well plates. Next, the virus inoculum was removed, and fresh medium was added to the culture. Cells were subsequently incubated at 37°C for 10 h before harvesting and analysis. UVC irradiation (300 J/m^2) was performed on 10-cm culture dishes of 293T cells (80% confluence) in a Stratalinker (Stratagene, La Jolla, CA). Cells were incubated 8 h at 37°C before harvesting and analysis.

Cell Transfections

For DNA transfection, cells were transfected using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) following the supplier's instructions. Cells were subsequently incubated at 37°C for 24 h before harvesting and analysis. For RNA transfection, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 0.5 μg of each bicistronic RNA. Cells were incubated at 37°C for 6 h before harvesting and analysis.

Enzymatic Activities

The luciferase activities were measured using the Dual Glo Luciferase kit (Promega, Madison, WI) and a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Briefly, 10 μl cell lysate was combined sequentially with Firefly and then *Renilla* luciferase-specific substrates according to the protocol supplied by the manufacturer. Light emission was measured 3 s after addition of each of the substrates and integrated over a 10-s interval.

RNA Interference (RNAi)

Small interfering RNA (siRNA) oligonucleotides against hnRNP A1 (A1-1: 5′-AAGGGAGGAAAUUUUGGAGGC-3′ [Patry *et al*., 2003]; A1-2: 5′-GCUCUUCAUUGGAGGGUUG-3′ [Bonnal *et al*., 2005]), PTB (PTB-P1: 5′-AACUUCCAUCAUUCCAGAGAA-3′ [Wollerton *et al*., 2004); PTB-Bis: 5′-AAGCCUCUUUAUUCUUUUCGG-3′), unr (5′-AAUGGUACUUCAGCAGCACUG-3′), a control siRNA (control: 5′-GGUCCGGCUCCCCCAAAUG-3′), and an siRNA targeting the RLuc open reading frame (ORF) (5′-GGGAUGAAUGGCCUGAUAUUGA-3′ [Sherrill *et al*., 2004]) were synthesized by Eurogentec (Seraing, Belgium). Transfections were performed on 30–50% confluent cells with a siRNA concentration of 200 nM. siRNAs were transfected in HeLa cells with Oligofectamine (Invitrogen) according to the manufacturer's recommendations. Western blot analysis was performed 48 h posttransfection.

UV Cross-linking, Immunoprecipitation, and Two-dimensional (2D) Gel Analysis

UV cross-linking experiments and immunoprecipitation of cross-linked hnRNP A1 with the 4B10 monoclonal antibody (mAb) were performed as described previously (Bonnal *et al*., 2005). Protein extracts (10 μg) were mixed with in vitro transcribed ^{32}P-labeled RNAs (150,000 cpm) in buffer GS (5 mM HEPES-KOH, pH 7.6, 30 mM KCl, 2 mM $MgCl_2$, 0.2 mM dithiothreitol, and 4% glycerol) for 10 min. The reaction mixtures were irradiated on ice with UV light (254 nm) in a Stratalinker (Stratagene) at 0.4 J/cm^2 at 10-cm distance. Five units of RNAse ONE (Promega) was then added, and the reaction mixtures were incubated for 45 min at 37°C. SDS gel loading buffer was added and the samples were boiled 2 min before fractionation on a 10% SDS-polyacrylamide gel. For immunoprecipitation of UV cross-linked proteins, the RNAse ONE-treated samples were diluted in 150 μl of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), precleared and mixed with 1 μl of anti-hnRNP A1 mAb (α4B10). The mixtures were allowed to rotate 1 h at 4°C. Then, 50 μl of protein A beads was added to the mixtures, and incubation was continued for 1 h at 4°C. After extensive washing of the beads, the bound proteins were eluted in SDS-loading buffer. The 2D gel electrophoresis analysis was performed with the ZOOM IPGRunner System from Invitrogen. Briefly ZOOM strips pH 3–10NL (Invitrogen) were rehydrated overnight in a rehydration buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 0.5% ZOOM carrier ampholytes, pH 3–10 (Invitrogen), 0002% bromphenol blue, and the samples. The ZOOM strips were then subjected to Isoelectric focusing following the manufacturer's instructions. For the second dimension NuPAGE Novex 4–12% Bis-Tris ZOOM gels (Invitrogen) were used. The cross-linked proteins were visualized by autoradiography after fixation and drying of the gel.

Streptavidine Acrylamide Precipitation with Biotinylated RNA

This experiment was performed as described previously (Vagner *et al*., 1995). One microliter of biotinylated RNA (200 ng/μl in solution containing 2 mg/ml calf liver tRNA (Roche Diagnostics, Mannheim, Germany) was incubated with 1 μl of a S-labeled PTB protein (translated in wheat germ extract) in 8 μl of KHN buffer (150 mM KCl, 20 mM HEPES, pH 7.9, 0.05% Nonidet P-40, and 0.2 mM dithiothreitol) for 1 h at 25°C. The mixture was then diluted with 500 μl of KHN buffer, transferred to a tube containing 30 μl of streptavidin acrylamide beads (Pierce Chemical, Rockford, IL), and incubated 1 h at 20°C. The beads were collected by centrifugation, washed four times with 1 ml of KHN buffer, resuspended in 10 μl of sample buffer, and boiled for 5 min. After centrifugation, the supernatant was loaded on to an SDS-polyacrylamide gel electrophoresis (PAGE) gel. The gel was exposed after electrophoresis and after a fluorography treatment using Amplify (GE Healthcare, Chalfont, St. Giles, United Kingdom).

Nitrocellulose Filter Binding Assays

Increasing amounts of a purified glutathione transferase (GST)-A1 were added to in vitro transcribed ^{32}P-labeled RNAs corresponding to the HRV or apaf-1 IRESs in a total volume of 10 μl of GS binding buffer containing 400 ng of yeast tRNA. The mixture was allowed to incubate 10 min at room temperature. Eight microliters of each binding reaction was applied on a presoaked nitrocellulose membrane on a slot dot apparatus (Hybrislot manifold; Invitrogen) under moderate suction. Each slot dot was washed with 200 μl of room temperature GS buffer, and the membranes were dried for 1 h at room temperature. The filters were exposed in a PhosphorImager cassette (GE Healthcare) for 3 h and revealed. The quantifications were performed with the ImageQuant version 1.1 software, and the data were corrected for the background (RNA retention without any added protein), which was <2%. The fraction of RNA bound was plotted against the protein concentration. Binding curves of three independent experiments were fitted by using SigmaPlot (Systat Software, Point Richmond, CA) to determine the apparent dissociation constants.

RNAse Protection Assay (RPA)

RNA was isolated from transfected cells with the use of TRIzol RNA reagent (Invitrogen) according to the manufacturer's protocol. The RPA was performed using the Ambion RPA III kit (Ambion, Austin, TX). Antisense RNA probes were generated by T7-polymerase transcription by using PCR fragments amplified from pCRHRVL with the primers described in Supplemental Figure S6 and Supplemental Table 2. In addition, actin cDNA was used as an internal control. The probes were radiolabeled with [^{32}P]UTP and ~60,000 cpm of each probe were hybridized together with 5 μg of total RNA per sample. The hybridized fragments were separated by polyacrylamide-urea gel electrophoresis and visualized by autoradiography. The gels were also exposed in a phosphorimager for quantification.

Western Blot Analysis

Protein analyses by Western blot on whole cell lysates were performed with standard protocols and a mAb against hnRNP A1 (4B10; Abcam, Cambridge, United Kingdom), a mAb against PTB (BB7; gift from Douglas Black, HHMI, University of California, Los Angeles), a polyclonal antibody against unr (gift from Hélène Jacquemin-Sablon, Inserm Bordeaux), or a polyclonal antibody against actin (Sigma-Aldrich, St. Louis, MO).

Immunocytochemistry and Immunofluorescence Experiments

Cell lines were grown on sterilized glass slides (Dako Denmark, Glostrup, Denmark). For immunocytochemistry, the slides coated with cells were fixed for 3 min in RCL2 fixative (AlphelysSA, Plaisir, France). Immunocytochemistry was then performed using a Techmate Horizon (Dako Denmark) slide processor. The primary antibody directed against hnRNPA1 (clone 4B10, dilution 1:6000; Abcam) or directed against the hemagglutinin (HA) epitope (Eurogentec) was incubated for 60 min and revealed using a streptavidin-biotin complex reagent (Envision; Dako Denmark). The slides were counterstained with hematoxylin. For immunofluorescence, cells were fixed in 3% paraformaldehyde for 15 min and permeabilized in 0.2% Triton for 5 min. Nonspecific binding sites were blocked by incubation with 0.1 M phosphate-buffered saline (PBS) and 1% bovine serum albumin for 30 min at room temperature. Subsequently, cells were incubated for 1 h with the anti-hnRNP A1 monoclonal primary antibody (4B10), followed by a 30-min incubation with an anti-mouse immunoglobulin G fluorescein isothiocyanate conjugate secondary antibody (Sigma-Aldrich). Cells were examined with a Leitz fluorescence microscope with a 63× objective.

Polysomal Fractionation Analysis and Northern Blot

293T cells (30 millions) were treated with 0.1 mg/ml cycloheximide (CHX) for 15 min at 37°C, washed twice with ice-cold PBS/CHX, and scraped in PBS/CHX. After centrifugation 5 min at 3000 rpm, the cell pellet was resuspended in 400 ml of LSB buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, and 100 U/ml RNAsine). After 10 strokes of Dounce homogeneization, 400 ml of LSB containing 0.2% Triton X-100 and 0.25 M sucrose was added. Cellular debris was removed by centrifugation. The lysate was layered on an 11.3-ml continuous sucrose gradient (15–50% sucrose in LSB buffer). After 120 min of ultracentrifugation at 38,000 rpm in a SW41-Ti rotor at 4°C, the fractions were collected with an ISCO (Lincoln, NE) density gradient fractionation system (Foxy Jr fraction collector coupled to UA-6 UV detector). The settings were as follows: pump speed, 0.75 ml/min; fraction time, 1.2 min/fraction; chart speed, 120 cm/h; and sensitivity of the OD254 recorder, 2. The absorbance at 254 nm was measured continuously as a function of gradient depth; 15 fractions of 0.9 ml were collected.

RNA from the polysomal fractions were extracted by phenol/chloroform (after proteinase K treatment) and analyzed by agarose gel and SYBR Green staining or Northern blot as described below. To detect mRNAs encoding apaf-1 or actin, we used [α-^{32}P]dCTP-labeled DNA PCR fragments (Prime-a-Gene labeling system; Promega) amplified with the following primers (Apaf-1: 5′-AGAGTGTTACAGATTCAGTAAT-3′ and 5′-TCTAGTATATGTCTGTATTCCA-3′; and Actin: 5′-GCGACGAGGCCCAGAGCA-3′ and 5′-TGCCAGGGCAGTGATCTC-3′).

RESULTS

HnRNP A1 Interacts with the HRV and the apaf-1 IRES

To investigate a possible direct interaction between hnRNP A1 and the HRV or apaf-1 IRESs, we used an UV cross-linking assay. Because hnRNP A1 has one of the most basic isoelectric points among mRNA binding proteins (Swanson and Dreyfuss, 1999; Figure 1Ai), migration of the cross-linked proteins was performed by two-dimensional gel electrophoresis, allowing for its easy separation from other mRNA binding proteins. ^{32}P-labeled FGF-2 IRES RNA was used as a control RNA in these experiments, because we demonstrated previously an interaction of hnRNP A1 with this IRES (Bonnal *et al*., 2005). We indeed observed a cross-linked RNA–protein complex migrating at the size (34 kDa) and isoelectric point (basic) of hnRNP A1 (Figure 1Aii). We next performed this experiment with four ^{32}P-labeled RNAs corresponding to the HRV, the encephalomyocarditis virus (EMCV), the apaf-1, and the bag-1 IRESs. The EMCV and the bag-1 IRESs were used as control RNAs, because they are not known to interact with hnRNP A1 (Pileur and Vagner, unpublished data). Although a cross-linked RNA–protein complex was found at the migration position of hnRNP A1 when using the HRV and the apaf-1 IRESs, no cross-linked RNA–protein complex was found for the EMCV and bag-1 IRESs (Figure 1Aiii).

We next examined whether the 34-kDa protein cross-linked to the HRV and apaf-1 IRESs was immunologically related to hnRNP A1 by immunoprecipitating the UV cross-linked RNA–protein complexes with the hnRNP A1-specific 4B10 mAb. The 34-kDa protein was detected after immunoprecipitation of the UV cross-linked proteins from both IRESs, but not from the EMCV IRES, showing that hnRNP A1 interacts directly and specifically with the HRV and apaf-1 IRESs (Figure 1B).

As a complementary approach to demonstrate an interaction between hnRNP A1 and both IRESs, we used an RNA affinity chromatography procedure in which biotinylated in vitro transcribed RNAs were incubated with a ^{35}S-labeled hnRNP A1 protein that was in vitro translated in rabbit reticulocyte lysate (RRL) (Figure 1C). Analysis of the complexes bound to streptavidin beads showed that the labeled full-length hnRNP A1 protein was retained on beads loaded with the HRV or apaf-1 IRESs (Figure 1C, compare lanes 4 and 5 with lane 1; ~10% of the input is retained), but it was very poorly retained on empty beads or on beads loaded with the EMCV IRES (Figure 1C, lanes 2 and 3; see also Supplemental Figure S1). Noteworthy, truncated hnRNP A1 proteins, resulting from spurious translation initiation or

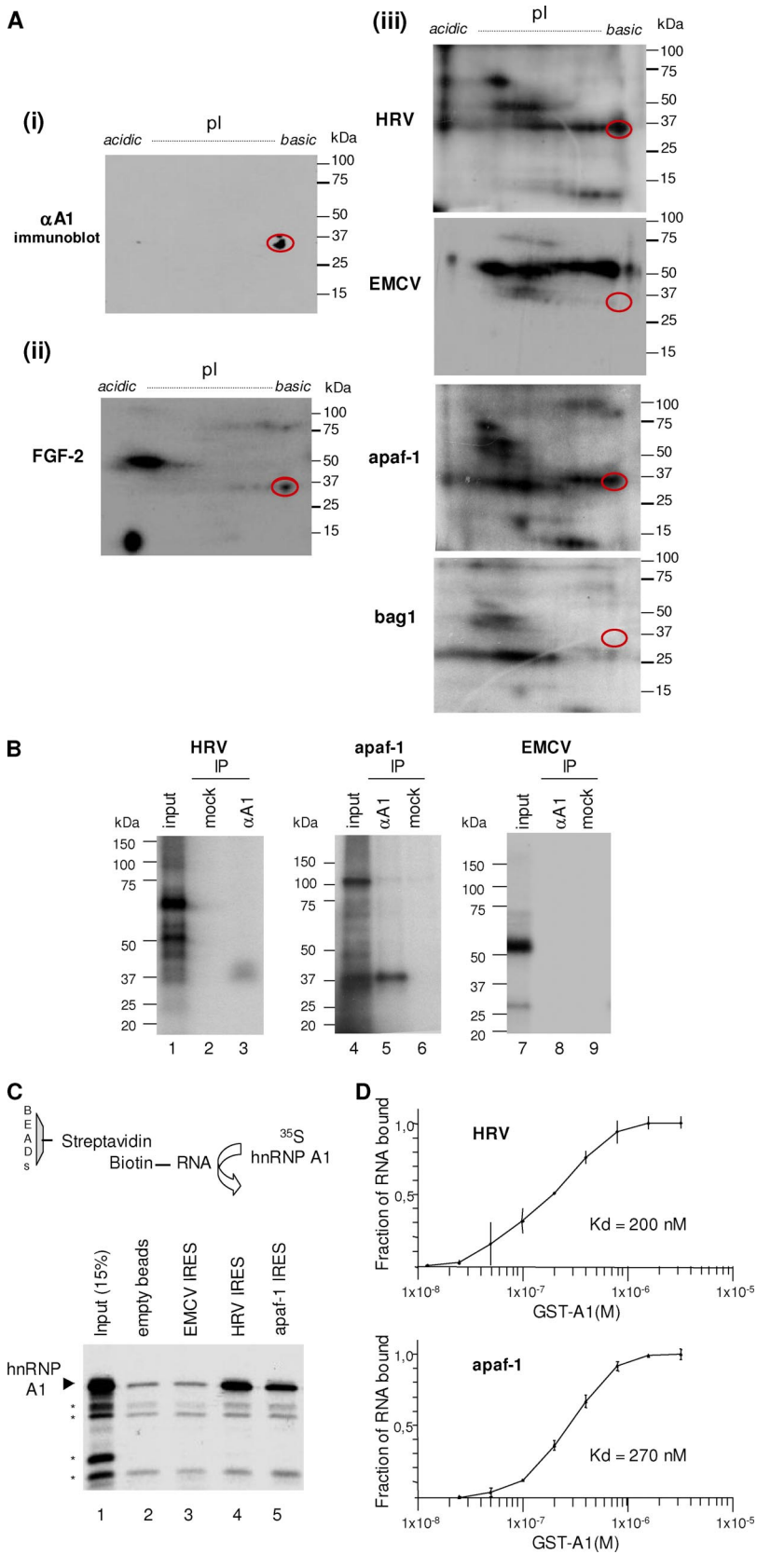

Figure 1. hnRNP A1 binds to the human apaf-1 and rhinovirus IRES. (Ai) Western immunoblot (αA1) analysis of hnRNP A1 after 2D gel electrophoresis of HeLa nuclear extract was done with the 4B10 mAb against hnRNP A1. (ii and iii) UV cross-linking of HeLa cell nuclear extracts (NE) with ^{32}P-labeled RNAs corresponding to the 5′ UTRs of the FGF-2 mRNA (ii) or to the HRV, EMCV, apaf-1, and bag1 mRNAs (iii). UV-cross-linked proteins were analyzed by 2D gel electrophoresis. Molecular-weight markers in kilodaltons are indicated at the right-hand side of each gel. pI represents the isoelectric point. The red circle indicates the position of hnRNP A1 migration. (B) UV cross-linking of HeLa NE with ^{32}P-labeled HRV, apaf-1 and EMCV 5′ UTR RNAs. The positions of protein molecular-weight markers in kilodaltons are indicated at the left-hand side of the gels. Immunoprecipitation (IP) of cross-linked RNA-protein complexes was performed with (αA1) or without (mock) the 4B10 mAb. (C) Streptavidin acrylamide bead precipitation of hnRNP A1 protein with biotinylated RNA. ^{35}S-labeled in vitro-translated hnRNP A1 protein was incubated with biotinylated RNAs corresponding to the HRV and apaf-1 5′ UTRs. The biotinylated RNA–^{35}S-protein complexes were then precipitated with streptavidin acrylamide beads and analyzed by SDS-PAGE. The RNAs used corresponded to the EMCV IRES (lane 3), the HRV IRES (lane 4), and the apaf-1 IRES (lane 5). Lane 1 corresponds to 15% of the input/assay; lane 2 corresponds to incubation of the proteins with the beads in the absence of RNA. Bands indicated with a star represent truncated forms of hnRNP A1 resulting from spurious translation initiation/termination events in RRL. Experiments were performed at least five times. (D) hnRNP A1 binding curve to the HRV and apaf-1 5′ UTR RNAs. Filter binding assays were carried out and evaluated as described in *Materials and Methods*. Filter-bound RNA is plotted as function of the protein concentration and corrected for the fraction of active protein. Curves were fitted to average data points of three independent experiments.

termination events frequently observed in RRL, were equally retained on any of the tested beads (Figure 1C). Together, these data confirm that hnRNP A1 is able to interact specifically with both IRESs. This experiment also

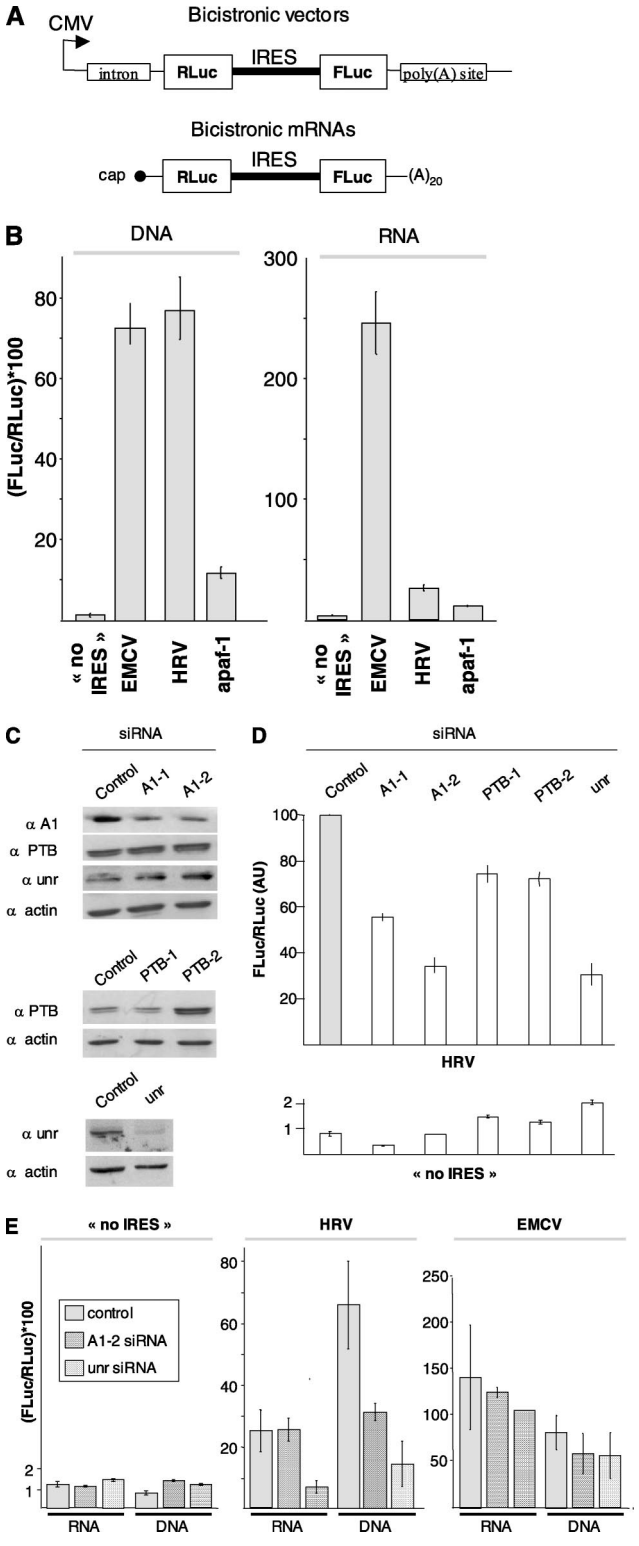

Figure 2. siRNA-mediated depletion of hnRNP A1 reduces HRV IRES activity. (A) Schematic representation of the bicistronic vectors or capped bicistronic mRNAs containing a 20-nucleotide-long poly (A) tail. CMV, cytomegalovirus promoter; RLuc, luciferase *Renilla* ORF; FLuc, luciferase firefly ORF. (B) The FLuc/RLuc activity ratio was measured in extracts of HeLa cells transfected with bicistronic vectors (DNA) (24 h posttransfection) or in vitro synthesized mRNAs (RNA) (6 h posttransfection). Bicistronic constructs containing the EMCV, the HRV, or the apaf-1 IRES were used. A no IRES construct was used as a control. The RLuc and FLuc values are shown in Supplemental Table S1. (C) The consequences of siRNA treatments were analyzed 48 h after siRNA transfection by Western immunoblotting with the indicated antibodies. α A1 corresponds to the monoclonal 4B10 antibody (Abcam), α PTB corresponds to a monoclonal Bb7 antibody provided by D. Black, α unr corresponds to a polyclonal antibody provided by H. Jacquemin-Sablon, and α actin corresponds to an actin polyclonal antibody (Sigma-Aldrich). The control lane corresponds to a transfection with a control siRNA. The RLuc and FLuc values are shown in Supplemental Table S2. (D) siRNA-mediated reductions in the expression of hnRNP A1, hnRNPI/PTB, or unr lead to various degrees of reduction in HRV IRES activity. The FLuc/RLuc ratio (AU, arbitrary units) driven by the HRV IRES or the no IRES construct were measured in extracts of HeLa cells transfected with bicistronic plasmids. The FLuc/RLuc ratio was arbitrarily set to 100 for extracts of HeLa cells transfected with the bicistronic plasmid containing the HRV IRES in the presence of the control siRNA. (E) siRNA-mediated reduction in the expression of hnRNP A1 reduces HRV IRES activity after DNA transfection, but not RNA transfection. The FLuc/RLuc ratio driven by the HRV IRES, the EMCV IRES or the no IRES construct was measured in extracts of HeLa cells transfected with bicistronic plasmids (DNA) or in vitro-synthesized mRNAs (RNA), and treated or not (control) with siRNAs targeting hnRNP A1 (A1 siRNA) or unr (unr siRNA). These experiments were performed at least three times.

allowed us to identify two mutants (consisting of only four to five substitutions) of the HRV IRES that had lost their interaction with hnRNP A1 (Supplemental Figure S1).

To further determine the binding characteristics of hnRNP A1 for both IRESs, we measured the apparent equilibrium dissociation constant (K_d) of hnRNP A1 for the IRES-containing RNA sequences by using a nitrocellulose filter binding assay. Binding experiments were carried out by incubating variable amounts of a recombinant *Escherichia coli*-produced GST-hnRNP A1 protein (GST-A1) with a constant amount of RNA. The apparent K_d of the binding reaction was estimated by determining the concentration of protein at which half-maximum binding was observed. We found that the K_d of recombinant GST-A1 protein is ~200 nM for the HRV IRES and 270 nM for the apaf-1 IRES (Figure 1D). Noteworthy, this affinity is comparable with the affinity defined between hnRNP A1 and the FGF-2 IRES in our previous work (Bonnal *et al*., 2005). Together, these data demonstrate that hnRNP A1 interacts specifically with both HRV and apaf-1 IRESs.

siRNA-mediated Knockdown of hnRNP A1 Expression Reduces HRV IRES Activity

To address the functional consequences of hnRNP A1 binding on these IRESs, we transfected bicistronic constructs containing an upstream *Renilla* luciferase gene (RLuc) and a downstream firefly luciferase gene (FLuc) in HeLa cells (Figure 2A). In such a bicistronic mRNA, the RLuc activity reflects cap-dependent translation, and it is expected to be proportional to the amount of RNA present in the cell, whereas the FLuc activity measures the IRES-dependent translation. Therefore, the FLuc/RLuc ratio represents the IRES activity normalized to the amount of RNA present in the cell. We found that the FLuc/RLuc ratio for the EMCV, the HRV, and the apaf-1 IRES-containing DNA constructs were, respectively, ~75-, 80-, or 12-fold the ratio obtained with the "no IRES" control (Figure 2B). Because the FLuc/RLuc ratio after DNA transfection may also be related to a nuclear event, such as the use of a cryptic promoter or splice site in the IRES sequence, we used siRNA to knock down transcripts containing the upstream (RLuc) coding region as a control experiment to detect cryptic monocistronic tran-

scripts (Sherrill *et al*., 2004) (Supplemental Figure S2). In this experiment intact bicistronic mRNAs containing both the RLuc and FLuc open reading frames are targeted by the RLuc siRNA, and therefore the levels of both RLuc and FLuc should be reduced comparably. If a cryptic nuclear event occurs that allows the production of monocistronic FLuc mRNAs, then the FLuc levels should not be reduced as much as RLuc levels after RLuc RNAi treatment. We found that the expression of FLuc was affected by a cryptic event present for the apaf-1 IRES, because the downstream (FLuc) reporter activity was not reduced in proportion to the upstream (RLuc) reporter activity after RNAi treatment; no cryptic nuclear events were detected for the HRV IRES (Supplemental Figure S2). We therefore concluded that apaf-1 IRES activity could not be measured after DNA transfection of our bicistronic vector construct. As a means to study the apaf-1 IRES, we synthesized capped and polyadenylated bicistronic mRNAs in vitro, which we transfected into the cytoplasm to avoid the effects of confounding nuclear events.

After transfection of bicistronic mRNAs, the FLuc/RLuc ratio for the EMCV, the HRV, and the apaf-1 IRES were, respectively, ~250-, 30-, and 2-fold the ratio obtained with the no IRES control (Figure 2B). The FLuc/RLuc ratio for both the HRV and the apaf-1 IRES was therefore lower (compared with the EMCV IRES) after RNA transfection, compared with DNA transfection. Importantly, this was not the case for the EMCV IRES-containing constructs, which showed that the lower FLuc/RLuc ratio after RNA transfection is not due to poor quality or design of the in vitro synthesized mRNA. The higher FLuc/RLuc ratio after DNA transfection of the apaf-1 IRES-containing construct was likely due to the previously identified cryptic nuclear event. After RNA transfection, the very low apaf-1 IRES activity was about twofold higher than the no IRES control mRNA. The role of hnRNP A1 on apaf-1 IRES-mediated translation could not be analyzed in these conditions.

Because no cryptic nuclear events were demonstrated for the HRV IRES (Supplemental Figure S2), the role of hnRNP A1 in HRV IRES-mediated translation was investigated. hnRNP A1 binding-deficient rhinovirus IRES mutants were affected for activity (Supplemental Figure S1), strongly suggesting a role of this interaction in HRV IRES function. The effect of siRNA-mediated knockdown of hnRNP A1 expression on HRV IRES activity after DNA transfection was analyzed with two different siRNAs targeting hnRNP A1, and it was compared with the decrease measured after siRNA-mediated knockdown of unr and PTB, two other ITAFs known to affect the HRV IRES (Hunt *et al*., 1999; Boussadia *et al*., 2003) (Figure 2, C and D). siRNA-mediated depletion of hnRNP A1 led to a two- to threefold decrease in HRV IRES activity (Figure 2D). Noteworthy, whereas siRNA-mediated reduction in the expression of unr led to a threefold decrease in HRV IRES activity (consistent with its demonstrated in vivo effect on HRV IRES-mediated translation; Boussadia *et al*., 2003), siRNA-mediated depletion of hnRNP I/PTB led to only a slight decrease in HRV IRES activity (Figure 2D) despite the demonstration of an in vitro role for PTB in HRV IRES-mediated translation (Hunt *et al*., 1999). However, the poor effect of PTB knockdown on HRV IRES-mediated translation may be due to an incomplete reduction of PTB expression (Figure 2C). Nevertheless, together these data indicate that hnRNP A1 is a novel ITAF for the HRV IRES.

Because hnRNP A1 resides primarily in the nucleus, we hypothesized that the low efficiency of the HRV IRES after RNA transfection may be due to the low level of hnRNP A1 in the cytoplasm. If this is the case, the HRV IRES activity of transfected bicistronic mRNA should be insensitive to siRNA-mediated knockdown of hnRNP A1 expression. Indeed, whereas the reduction in hnRNP A1 levels decreased HRV IRES activity after DNA transfection, it had no effect on IRES activity after RNA transfection (Figure 2E). As expected, siRNA-mediated knockdown of unr reduced IRES activity after both DNA and RNA transfections, consistent with its cytoplasmic localization (Figure 2E). Thus, the relocalization of hnRNP A1 to the cytoplasm may be a positive switch to control HRV IRES-mediated translation.

Cytoplasmic-relocalized hnRNP A1 Activates HRV IRES-mediated Translation after Rhinovirus Infection

To investigate the role of cytoplasmic hnRNP A1 in HRV IRES activity, we artificially expressed a cytoplasmically restricted mutant of hnRNP A1 lacking its carboxy-terminal M9 shuttling domain (hnRNP A1 ΔM9) (Izaurralde *et al*., 1997). To avoid problems associated with poor efficiency of cotransfection of the hnRNP A1 ΔM9-encoding plasmid and the bicistronic reporter RNA, we inserted the hnRNP A1 ΔM9 ORF in place of the first cistron of the bicistronic mRNA (Figure 3A). A control RNA that did not produce the hnRNP A1 ΔM9 protein was generated by replacing the hnRNP A1 ΔM9 ORF with a chloramphenicol acetyl transferase (CAT) ORF. As a control for the specificity of the effect of hnRNP A1 on the HRV IRES, we also performed the experiment with the M5 mutant of the HRV IRES that is strongly reduced in its ability to interact with hnRNP A1, as judged by the RNA affinity chromatography procedure described in Figure 1B (Figure 3B; also see Supplemental Figure S1). The amount of transfected bicistronic RNAs was assessed using an RNase protection assay, and it was found to be equivalent (Figure 3C, top). The proper synthesis and cytoplasmic localization of the hnRNP A1 ΔM9 mutant was confirmed by Western blot and immunocytochemistry by virtue of an amino-terminal HA tag (Figure 3C, bottom, and D). Expression of the hnRNP A1 ΔM9 protein, but not the CAT protein, increased the luciferase activity of the bicistronic reporter containing the wild-type HRV IRES, whereas it did not have any effect on the luciferase activity driven by the M5 HRV IRES mutant (Figure 3E). Furthermore, expression of the full-length hnRNP A1 protein did not increase the luciferase activity of the bicistronic reporter containing the wild-type HRV (Supplemental Figure S3). Thus, the expression of a cytoplasmic hnRNP A1 specifically activates HRV IRES-mediated translation.

The role of the cytoplasmic relocalization of hnRNP A1 on HRV IRES-mediated translation was further investigated after rhinovirus infection. Indeed, as expected from Gustin and Sarnow (2002), we found that rhinovirus infection led to a partial cytoplasmic relocalization of hnRNP A1 (Figure 4A). We transfected a bicistronic mRNA containing the HRV IRES in mock- or rhinovirus-infected cells. Compared with the HRV IRES activity in mock-infected cells, we observed a slight but significant increase of HRV IRES activity upon rhinovirus infection (Figure 4B). This effect was specific, because the EMCV IRES activity was unchanged upon rhinovirus infection (Figure 4B). Because several nuclear proteins are relocalized to the cytoplasm upon rhinovirus infection (Gustin and Sarnow, 2001), we wondered whether the stimulatory effect was directly linked to hnRNP A1 relocalization to the cytoplasm. We therefore performed siRNA-mediated knockdown of hnRNP A1 in mock or infected cells. We observed that rhinovirus infection did not cause an increase in HRV IRES activity in cells in which the expression of hnRNP A1 was reduced (Figure 4B). This shows that

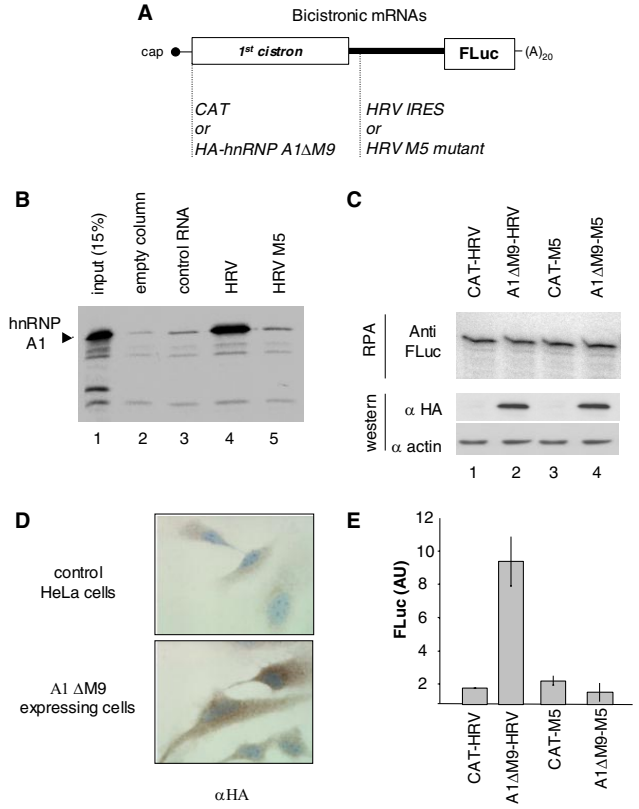

Figure 3. Expression of a cytoplasmically restricted hnRNP A1 mutant leads to enhanced HRV IRES activity. (A) Schematic representation of the bicistronic reporter RNAs used to express a HA-tagged cytoplasmically restricted hnRNP A1 mutant (A1ΔM9) and a control protein (CAT), and to quantify rhinovirus IRES-mediated translation. The wild-type HRV IRES or the M5 mutant of the HRV IRES, which is reduced in its ability to interact with hnRNP A1 (see Supplemental Figure 1 and B), were inserted into the intercistronic space. (B) Streptavidin acrylamide precipitation of hnRNP A1 protein with biotinylated RNA. The RNAs used corresponded to a control RNA (lane 3), the wild-type HRV 5′ UTR (lane 4) or the M5 mutant of the HRV 5′ UTR (lane 5). Lane 1 corresponds to 15% of the input/assay; lane 2 corresponds to incubation of the proteins with the beads without RNA (C) Top, RPA using an antisense probe for the FLuc ORF (anti-FLuc). Bottom, Western-blot analysis of the expression of the mutant cytoplasmic hnRNP A1 by using an antibody against the HA tag (α HA). An actin Western blot was used as a loading control. (D) Immunocytochemistry experiments in HeLa cells were performed with the HA-tag antibody. (E) FLuc activity was measured in extracts of HeLa cells transfected with the various mRNAs, as indicated below the graph. This activity was normalized to the amount of transfected mRNAs measured by RPA experiments. The FLuc activity was arbitrarily set to 1 for the conditions corresponding to the transfection of the control CAT-HRV mRNA. These experiments were repeated three times for D, four times for C and E, and five times for B.

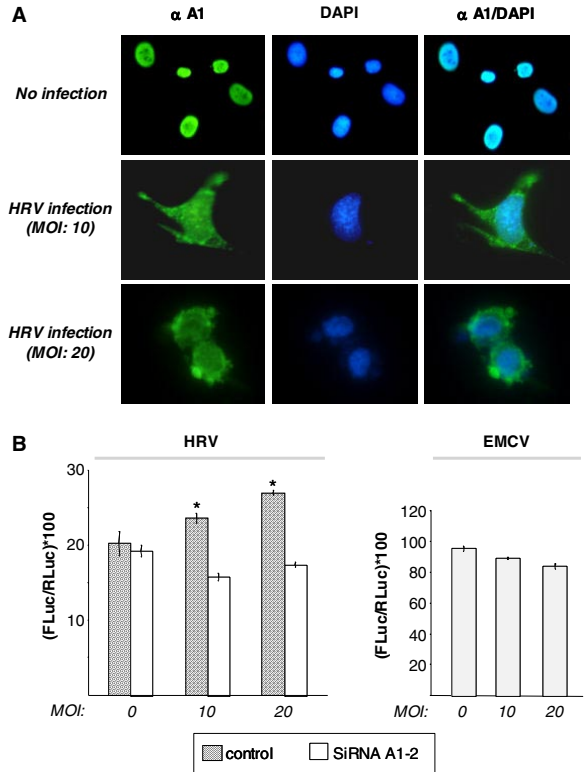

Figure 4. HRV IRES activity is stimulated upon rhinovirus infection. The human rhinovirus was added at a multiplicity of infection (MOI) of 0, 10, or 20 as indicated. After 1-h incubation, the virus inoculum was removed, and fresh medium was added to the culture. Cells were subsequently incubated for 10 h before harvesting and analysis. (A) hnRNP A1 accumulates in the cytoplasm in rhinovirus-infected HeLa cells. Immunostaining of noninfected or HRV-infected HeLa cells for hnRNP A1 (αA1) and the nuclear marker 4,6-diamidino-2-phenylindole (DAPI). The merged images (αA1/DAPI) show the presence of hnRNP A1 outside the nucleus in HRV-infected cells. (B) The FLuc/RLuc ratio was measured in extracts of HeLa cells transfected with in vitro synthesized bicistronic mRNAs containing either the HRV or the EMCV IRES. RNA transfections were performed 2 h before infection, whereas hnRNP A1 siRNA transfection was performed 48 h before infection. The RLuc and FLuc values are shown in Supplemental Table S3. This experiment was performed on three independent occasions (*$p < 0.04$).

cytoplasmic relocalization of hnRNP A1 induced by rhinovirus infection promotes HRV IRES-mediated translation.

Cytoplasmic-relocalized hnRNP A1 Inhibits apaf-1 IRES-mediated Translation during UVC Irradiation

We next investigated the involvement of hnRNP A1 in apaf-1 mRNA translation, because apaf-1 IRES-mediated translation is activated after UVC irradiation in human embryonic kidney (293T) cells (Ungureanu *et al*., 2006) and hnRNP A1 is redistributed to the cytoplasm after UVC irradiation (Figure 5A; as expected from van der Houven van Oordt *et al*., 2000). To first confirm the effect of UVC irradiation on endogenous apaf-1 mRNA translation, we examined the polysomal association of this mRNA in 293T cells. Polysome gradients were prepared from untreated cells or cells irradiated with a UVC dose of 300J/m^2, a dose previously shown to induce 50% cell death (Ungureanu *et al*., 2006). UVC irradiation caused a large decrease in polysomal mRNAs and a corresponding increase in free ribosomes and ribosomal subunits, reflecting a global reduction in translation initiation efficiency (Figure 5B). Northern blot analysis showed an UVC-dependent decrease in the presence of actin mRNA in the fractions containing translationally engaged polysomes (Figure 5Bi and ii, and C), consistent with the general translation defect. In spite of this, Northern blot analysis of the apaf-1 mRNA revealed a UVC-dependent shift of the polysomal distribution of this mRNA toward the polysomal fractions (Figure 5Bi and ii, and C) showing that the apaf-1 mRNA was indeed translated better after UVC irradiation, consistent with our previous results showing a

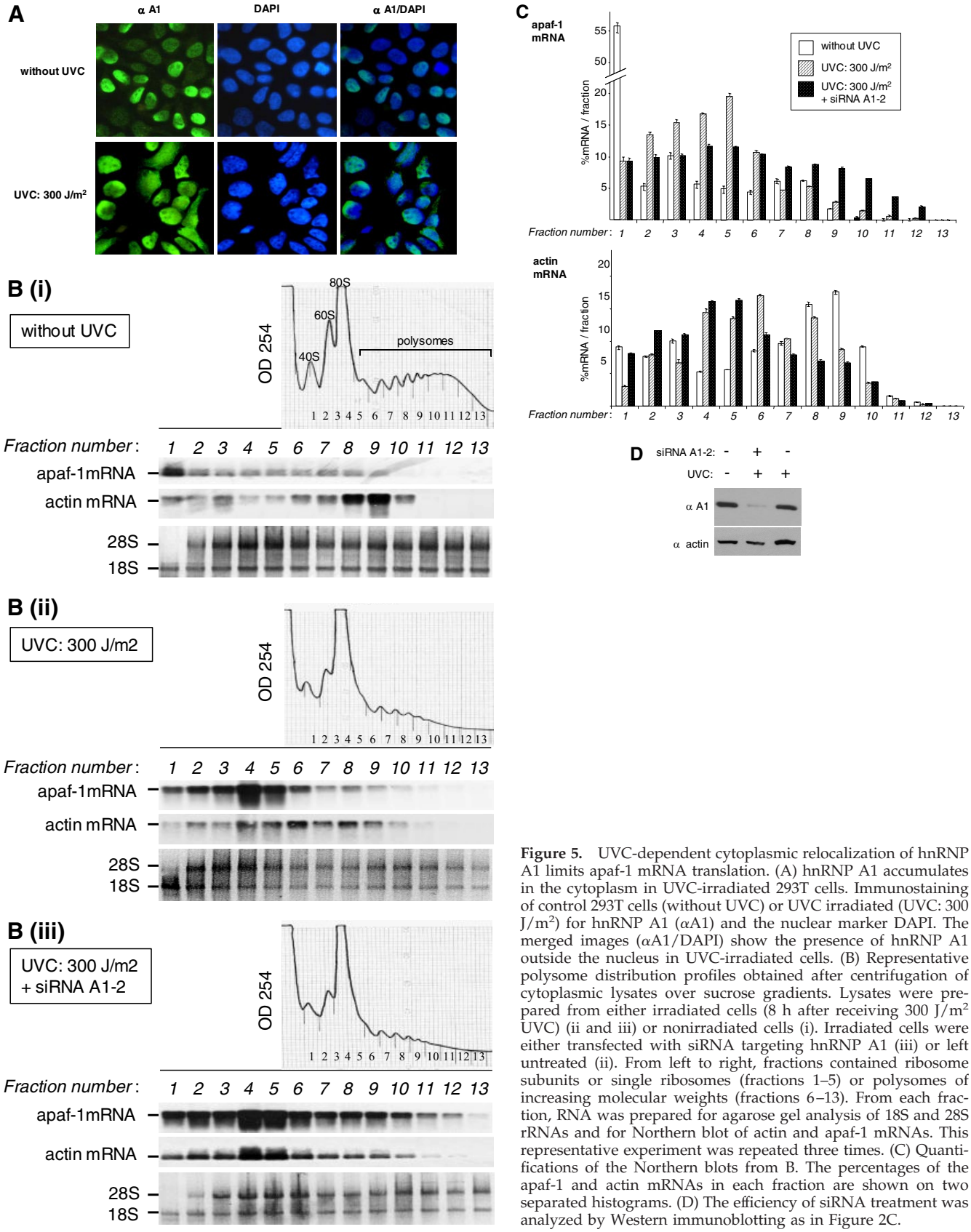

Figure 5. UVC-dependent cytoplasmic relocalization of hnRNP A1 limits apaf-1 mRNA translation. (A) hnRNP A1 accumulates in the cytoplasm in UVC-irradiated 293T cells. Immunostaining of control 293T cells (without UVC) or UVC irradiated (UVC: 300 J/m^2) for hnRNP A1 (αA1) and the nuclear marker DAPI. The merged images (αA1/DAPI) show the presence of hnRNP A1 outside the nucleus in UVC-irradiated cells. (B) Representative polysome distribution profiles obtained after centrifugation of cytoplasmic lysates over sucrose gradients. Lysates were prepared from either irradiated cells (8 h after receiving 300 J/m^2 UVC) (ii and iii) or nonirradiated cells (i). Irradiated cells were either transfected with siRNA targeting hnRNP A1 (iii) or left untreated (ii). From left to right, fractions contained ribosome subunits or single ribosomes (fractions 1–5) or polysomes of increasing molecular weights (fractions 6–13). From each fraction, RNA was prepared for agarose gel analysis of 18S and 28S rRNAs and for Northern blot of actin and apaf-1 mRNAs. This representative experiment was repeated three times. (C) Quantifications of the Northern blots from B. The percentages of the apaf-1 and actin mRNAs in each fraction are shown on two separated histograms. (D) The efficiency of siRNA treatment was analyzed by Western immunoblotting as in Figure 2C.

more efficient production of the Apaf-1 protein, as judged by Western blot analysis (Ungureanu *et al.*, 2006).

To determine the possible contribution of hnRNP A1 on apaf-1 IRES activity after UVC irradiation, we performed a

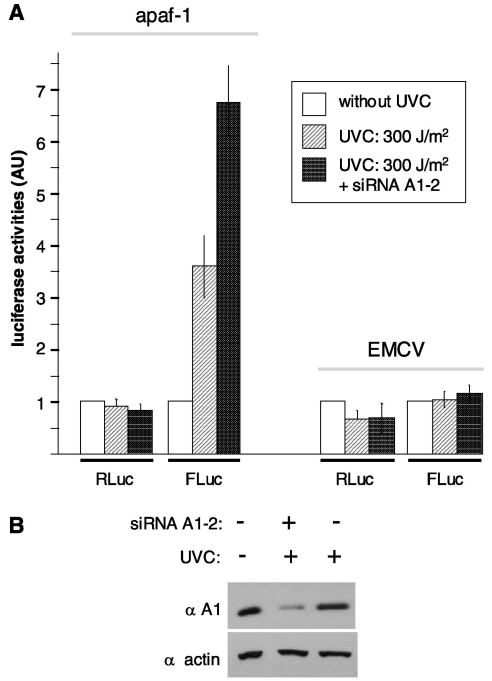

Figure 6. UVC-dependent cytoplasmic relocalization of hnRNP A1 limits apaf-1 IRES activity. (A) The FLuc and RLuc activities of transfected bicistronic mRNAs containing the apaf-1 and EMCV IRES were measured in extracts of 293T cells left untreated, irradiated with UVC, or irradiated with UVC after siRNA-mediated depletion of hnRNP A1. (B) The efficiency of siRNA treatment was analyzed by Western immunoblotting as in Figure 2C. This set of experiments was repeated at three different occasions.

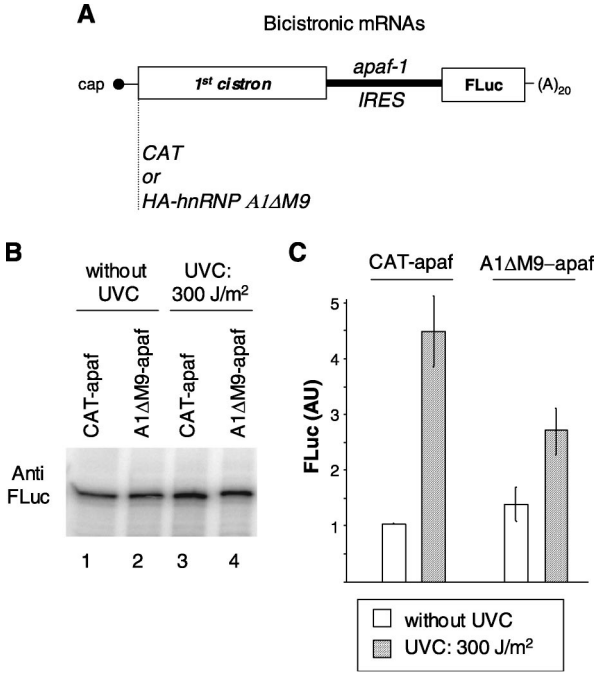

Figure 7. Expression of a cytoplasmically restricted hnRNP A1 mutant limits the UVC-triggered activation of apaf-1 IRES activity. (A) Schematic representation of the bicistronic reporter RNAs used to express a HA-tagged cytoplasmically restricted hnRNP A1 mutant (A1ΔM9) or a control protein (CAT) and quantify apaf-1 IRES-mediated translation. (B) RPA using an antisense probe for the FLuc ORF (anti-FLuc). (C) The FLuc activities were measured in extracts of transfected 293T cells that were UVC-irradiated or nonirradiated, as indicated. The two mRNAs were used, as indicated. This activity was normalized to the amount of transfected mRNAs measured by RPA experiments. The FLuc activity was arbitrarily set to 1 for the condition corresponding to the transfection of the control CAT-apaf mRNA in non-UVC exposed cells. This experiment was repeated three times.

siRNA-mediated knockdown of hnRNP A1 in UVC-irradiated cells (Figure 5D). We observed no change in the general polysomal profile between UVC-irradiated cells and UVC-irradiated cells in which the level of hnRNP A1 was reduced, showing that hnRNP A1 does not affect general translation initiation (Figure 5Bii and iii, and C). Indeed, the distribution of the actin mRNA, as judged by Northern blot, was not changed by hnRNP A1 knockdown (Figure 5Bii and iii, and C). However, we observed a shift of the apaf-1 mRNA to more heavy polysomal fractions in UVC-irradiated cells in which hnRNP A1 was knocked down (Figure 5Bii and iii, and C), showing that hnRNP A1 blocks translation initiation of the apaf-1 mRNA after UVC irradiation. Therefore, hnRNP A1 is not a factor that contributes to the UVC-dependent translational activation of the apaf-1 mRNA, but rather it limits its translation.

To analyze whether the blockade in translation initiation occurs through inhibition of apaf-1 IRES activity, we transfected bicistronic mRNAs containing the apaf-1 IRES into 293T cells, and we measured the luciferase activities after UVC irradiation (Figure 6A). A three- to fourfold UVC-dependent increase in the FLuc activity was observed without any significant change in the RLuc activity (Figure 6A). This effect was specific, because we did not observe changes in luciferase activities of a bicistronic mRNA containing the EMCV IRES (Figure 6). Furthermore, and consistent with the results obtained for the polysomal distribution of the apaf-1 mRNA (Figure 5), we observed an additional increase in the FLuc activity after a siRNA-mediated reduction of hnRNP A1 levels (Figure 6B) in UVC-irradiated cells without any change in the RLuc activity (Figure 6A). Together, these results demonstrate that apaf-1 IRES activity is increased after UVC irradiation but that the UVC-dependent relocalization of hnRNP A1 to the cytoplasm limits this stimulatory effect.

If this is indeed the case, the artificial expression of a cytoplasmically restricted hnRNP A1 protein mutated in its M9 domain should reduce apaf-1 IRES activity. We used a similar approach as described in Figure 3 for the HRV IRES. We generated constructs containing an upstream cistron corresponding to the hnRNP A1 ΔM9 protein or a control CAT protein and a downstream cistron corresponding to the FLuc ORF. The apaf-1 IRES was inserted into the intercistronic space (Figure 7A). The amount of transfected bicistronic RNAs was determined using an RNAse protection assay in untreated and UVC-irradiated cells (Figure 7B). Whereas a four- to fivefold increase in apaf-1 IRES activity was observed after UVC irradiation of cells transfected with the bicistronic mRNA containing the control CAT ORF, only a two- to threefold activation was observed after UVC irradiation of cells transfected with the bicistronic mRNA producing the mutant hnRNP A1 protein localized in the cytoplasm (Figure 7C). This demonstrates that apaf-1 IRES activity is reduced by cytoplasmic hnRNP A1.

DISCUSSION

The role in translational control of a class of predominantly nuclear mRNA binding proteins involved in splicing regu-

lation has recently emerged as a common theme in the field of gene expression. However, little is known about the relationship between the subcellular localization of these *trans*-acting factors and their ability to regulate translation. We have shown here that hnRNP A1, an abundant mRNA binding protein present in the nucleus of mammalian cells, is a translation *trans*-acting factor that modulates the IRES activity of two IRES-containing mRNAs. Three sets of data have allowed us to demonstrate that hnRNP A1 promotes IRES activity of the HRV mRNA but that it inhibits IRES-mediated translation of the apaf-1 mRNA. First, we have shown that hnRNP A1 binds specifically and with an affinity ranging from 200 to 270 nM to both IRESs (Figure 1). Additionally, we have provided evidence that expression of a cytoplasmically restricted mutant of hnRNP A1 deleted of its M9 carboxy-terminal shuttling domain either stimulates HRV IRES activity (Figure 3) or inhibits apaf-1 IRES activity (Figure 7), as measured after transfection of bicistronic reporter mRNAs containing these IRESs. Finally, siRNA-mediated knockdown of hnRNP A1 expression results in decreased HRV IRES activity (Figure 2) and increased IRES-mediated apaf-1 mRNA translation after UVC irradiation (Figure 6). Importantly, the IRES activities were generally evaluated after transfection of in vitro synthesized mRNAs, ensuring that our results reflect a true IRES activity and not the use of cryptic promoters or splice sites that would have been activated upon DNA transfection (see Supplemental Figure S2 for the apaf-1 IRES). Interestingly, we have recently shown that hnRNP A1 is an IRES *trans*-acting factor that activates translation of the FGF-2 mRNA (Bonnal *et al*., 2005) but inhibits translation of the XIAP (X-linked inhibitor of apoptosis) mRNA (Lewis *et al*., 2007). The role of hnRNP A1 as an ITAF now extends to four mRNAs, and we predict that it controls IRES-mediated translation of many more genes. The ability of hnRNP A1 to be either an activator or an inhibitor of IRES function (depending on its target IRES) may be related to its ability to catalyze RNA–RNA annealing (Kumar and Wilson, 1990; Munroe and Dong, 1992), because structural remodeling of the 5′ UTR may either potentiate or destroy the IRES. Alternatively, hnRNP A1 may compete with other ITAFs (either positive or negative regulators) for binding to specific IRES elements and thus regulate IRES activity in this manner.

Because hnRNP A1 is a predominantly nuclear protein, but it is able to relocalize in the cytoplasm in a regulated manner (i.e., picornavirus infection [Gustin and Sarnow, 2001] or stress stimuli such as osmotic shock or UVC irradiation in a p38 mitogen-activated protein kinase (MAP)-dependent pathway [van der Houven van Oordt *et al*., 2003]), the expectation is that this relocalization has a consequence for translational control. Two hypotheses could explain how this relocalization affects translational control. First, hnRNP A1 is preassembled with its target mRNA in the nucleus and is transported together with the mRNA into the cytoplasm, where it subsequently affects translation. In this case, translational control would be an indirect consequence of the cytoplasmic relocalization. Second, hnRNP A1 binds to its target mRNA in the cytoplasm and the cytoplasmic redistribution of hnRNP A1 constitutes a direct switch to control translation. Here, we have provided data that support this second hypothesis from our study of IRES-mediated translational control of two different mRNAs (HRV and apaf-1) that are regulated by two different stimuli (viral infection and UVC irradiation), raising the possibility that our conclusions may be generalized to other mRNAs.

Concerning the human rhinovirus, we have found that the HRV IRES is less active after RNA transfection than DNA transfection (Figure 2). We reasoned that this weaker activity after RNA transfection might be due to the lack of a sufficient amount of hnRNP A1 in the cytoplasm. We have indeed demonstrated that HRV IRES activity after RNA transfection is insensitive to siRNA-mediated knockdown of hnRNP A1 expression (Figure 2), but it is stimulated upon cytoplasmic relocalization of hnRNP A1 (Figure 3 and 4). As a way to force the cytoplasmic relocalization of hnRNP A1, we have either produced a mutant hnRNP A1 protein deleted of its carboxy-terminal M9 shuttling domain (Figure 3) or used physiological conditions, such as the infection by the rhinovirus (Figure 4), which lead to hnRNP A1 redistribution to the cytoplasm due to inhibition of its nuclear import (Gustin and Sarnow, 2001, 2002). Furthermore, rhinovirus infection has been recently shown to induce phosphorylation and activation of the p38 MAP kinase (Dumitru *et al*., 2006). The cytoplasmic relocalization of hnRNP A1 after rhinovirus infection may therefore also be dependent on the p38 MAP kinase pathway as it is during stress conditions (van der Houven van Oordt *et al*., 2003). Numerous reports have demonstrated that IRES elements have important functions in the viral life cycle, mostly to ensure efficient viral translation when components of the host translation machinery are limited due to virus-induced modifications or host-induced antiviral responses, such as the phosphorylation of eukaryotic initiation factor 2α (Hellen and Sarnow, 2001; Vagner *et al*., 2001). However, no evidence exists to support a direct positive influence on virus IRES-mediated translation after infection. Here, we have shown that rhinovirus infection exerts a direct positive switch to control its IRES-mediated translation through relocalization of an ITAF. Several other ITAFs have been described for the rhinovirus IRES, including unr, hnRNP I/PTB, and hnRNP E2 (Hunt *et al*., 1999; Walter *et al*., 1999; Boussadia *et al*., 2003). However, the in vivo role of these ITAFs in HRV IRES function after rhinovirus infection, as well as their respective role in IRES function in general, may warrant further investigations. Interestingly, we have not been able to detect strong and conclusive interactions between hnRNP A1 and either unr or PTB (data not shown), excluding the possibility of the existence of a multimeric complex involved in IRES function.

Concerning the apaf-1 gene, we have forced the cytoplasmic redistribution of hnRNP A1 by either producing a mutant hnRNP A1 protein deleted of its carboxy-terminal M9 shuttling domain or by using UVC irradiation. We have found that hnRNP A1 limits the UVC-dependent translational activation of endogenous apaf-1 mRNA translation (Figure 5) and apaf-1 IRES activity (Figures 6 and 7). Therefore, hnRNP A1 is not a factor that contributes to the UVC-dependent translational activation of the apaf-1 mRNA. The characterization and/or identification of such factors warrants further investigation. Nevertheless, our study demonstrates that cytoplasmic redistribution of hnRNP A1 exerts a direct switch to control translation of the proapoptotic apaf-1 mRNA. This is consistent with the recently reported role of hnRNP A1 in the stress response, underscored by the observation that cells lacking hnRNP A1 exhibit decreased viability rates during stress (Guil *et al*., 2006). This may also be consistent with the proapoptotic effect of siRNA-mediated reduction in hnRNPA1/A2 proteins observed in various cell types, although in that case, the proapopototic effect was found to be associated with a change in the distribution of the lengths of telomeric G-tails (Patry *et al*., 2003, 2004). Very interestingly, ectopic expression of a nuclear-restricted hnRNP A1 mutant was shown to enhance the susceptibility to apoptosis (Iervolino *et al*., 2002). The antiapoptotic func-

tion of hnRNP A1 may therefore be linked to its cytoplasmic function in translational control, in part through the inhibition of the apaf-1 IRES activity demonstrated in this study.

The results presented in this study also imply that UVC-triggered translational control can be mediated by an IRES-based mechanism and by hnRNP A1. This demonstrates that cellular IRESs behave as translational enhancers elements regulated by specific *trans*-acting mRNA binding proteins in given physiological conditions. It will be of interest to investigate UVC-induced IRES-dependent and/or hnRNP A1-dependent translational control of other mRNAs, including the 17 mRNAs that are translationally induced by UVC irradiation, which were identified in human RKO colorectal carcinoma cells (Mazan-Mamczarz *et al*., 2005). This may eventually define hnRNP A1-dependent IRESs as important elements in the UVC-triggered apoptotic pathway.

Last, the control of translation initiation through relocalization of an mRNA binding protein may occur more generally, because several reports demonstrate a regulated relocalization of members of the hnRNP family involved in translation. We have recently shown that hnRNP A1 is a negative regulator of XIAP mRNA translation during osmotic shock and that a mutant cytoplasmic hnRNP A1 exerts a more potent inhibitory effect on XIAP IRES translation than the wild-type hnRNP A1 protein (Lewis *et al*., 2007). Serum stimulation or constitutive activation of the extracellular signal-regulated kinase kinase 1 results in phosphorylation and cytoplasmic accumulation of hnRNP K (Habelhah *et al*., 2001). Phosphorylation-dependent cytoplasmic accumulation of hnRNP K is required for its ability to silence LOX mRNA translation (Habelhah *et al*., 2001). However, it has not been demonstrated whether hnRNP K binds the LOX mRNA in the nucleus or in the cytoplasm. hnRNP I (PTB) is an IRES *trans*-acting factor involved in translation regulation of many IRES-containing mRNAs (Valcarcel and Gebauer, 1997; Spriggs *et al*., 2005; Bushell *et al*., 2006). Interestingly, it was found that direct protein kinase A phosphorylation of PTB modulates its nucleocytoplasmic distribution (Xie *et al*., 2003). In all these cases, it remains to be determined whether the cytoplasmic relocalization directly controls cytoplasmic translation. A large-scale analysis of mRNA targets bound by translation *trans*-acting factors able to relocalize in a regulated manner in the cytoplasm will undoubtedly identify genes regulated at the translational level through relocalization of predominantly nuclear mRNA binding proteins.

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